MEGAKARYOCYTE DIFFERENTIATION FACTOR

BACKGROUND OF THE INVENTION

5   1.   Field of the Invention

The present invention relates to a megakaryocyte differentiation factor, gene coding for the factor and a process for production thereof.   The megakaryocyte differentiation factor is useful as a

10   hemopoietic stimulating factor for megakaryocyte-platelet linease.

2.   Description of Related Art

It is well known that various hemopoietic factors inducing the growth and differentiation of blood

15   cells are involved in a process from hemopoietic stem cells to mature blood cells.

Although the life time of platelet is as short as 9 to 10 days, concentration of platelets in the blood is maintained rather constant during the stationary

20   state.   Moreover, when the number of platelets is artificially reduced by one of various available method in an experimental animals, the number of the platelets recovers in the blood in a few days.   From these facts, it is supposed that factors which stimulate formation of

25   platelets are present, and so far a great effort has been made to identifycation of the factors.

It is considered that at least two regulatory factors are involved in the megakaryocyte-platelet hemopoietic lineage.   The first factor by itself

30   stimulates formation of megakaryocyte colonies and is called a megakaryocyte colony stimulating factor.   The

second factor by itself does not have an activity to stimulate formation of megakaryocyte colonies, but in combination with the first factor, increases the number of megakaryocyte colonies and stimulates the differentiation thereof, and is called a megakaryocyte potentiator.

The former includes interleukin 3, and granulocyte/macrophage colony stimulating factor, and the latter includes erythropoietin, macrophage colony stimulating factor, interleukin 6, 7 and 11, LIF, and the like. Some of these factors actually exhibit in vivo effect of increasing the number of platelets or shortening the time required to recover the number of platelets (Hideaki Mizoguchi: Tanpakushitsu Kakusan Koso 36, 1195, 1991 in Japanese).

However, most of these factors exhibit a diversity of biological activities other than participation in differentiation of blood cells in various hemopoietic lineages including differentiation in megakaryocyte-platelet lineages. For example, although IL-6 and IL-11 actually exhibit in vivo thrombopoietic action, they stimulate production of acute phase protein, and in severe cases, cause cachexia. Moreover, IL-6 is accompanied with various clinical problems; for example, it is possible for IL-6 to stimulate the growth of mesangium cells in the kidney resulting in renal failure (Tadashi Matsuda et al., Tanpakushitsu Kakusan Koso, 36, 1184, 1991 in Japanese). In addition, since IL-6 does not exhibit a high blood level during a thrombcytopenic phase, it is not considered as a physiological factor.

Platelets play an important role in a

hemostatic mechanism. Diseases involving decrease of platelets (Fanconi's syndrome, megakaryocytic thrombocytopenia, aplastic anemia, and the like) are clinically dangerous, and in particular hemorrhaging cannot be controlled. Therefore, it is considered that isolation and identification of a factor which stimulates production of platelets is useful to prevent the above-mentioned danger.

Currently, bone marrow transplantation is becoming a powerful therapeutic means for treating leukenia etc., and the ratio of successful cases is increasing through the use of cytokines such as erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF) etc. At present a problem in the bone marrow transplantation is a decrease in the number of platelets, and if a thrombopoietic factor is available, it is expected that the ratio of successful cases will increase and a period of hospitalization will be shortened. Not only hemopoietic diseases but also thrombocytopenia in chemotherapy and radio isotopic therapy of cancers may be controlled by thrombopoietin.

The present inventors, considering the various above-mentioned difficulties with known factors, carried out various research to find a factor which stimulates production of platelets and is effective for treatment of patients having thrombocytopenia or insufficient platelet function, and as a result, the present inventors found a novel factor which stimulates differentiation of megakaryocytes, cloned a gene coding for said factor, constructed an expression vector, and succeeded in expressing the gene to produce said factor.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a megakaryocyte differentiation factor having the following properties;

    (1)   stimulating differentiation of megakaryocytes;

    (2)   exhibiting a molecular weight of 55 to 57 kD as determined by gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and having no intermolecular disulfide linkage;

    (3)   exhibiting an isoelectric point of 6.5 ± 0.5; and

    (4)   having at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9 in the Sequence Listing.

The present invention also provides a gene coding for the megakaryocyte differentiation factor.

The present invention further provides an expression vector comprising the gene coding for the megakaryocyte differentiation factor.

The present invention moreover provides a host transformed with the expression vector.

The present invention still further provides a process for production of the megakaryocyte differentiation factor using said host.

BRIEF EXPLANATION OF DRAWINGS

Figure 1 shows an elution profile of protein (A280 ●) and a megakaryocyte differentiation factor (acetylcholine esterase activity o) from a Q-Sepharose column, wherein the elution was carried out by NaCl concentration gradient (0 to 1.0M) to obtain fractions 1 to 120.

Fig. 2 shows an elution profile of protein

(A280 ●) and a megakaryocyte differentiation factor (acetylcholine esterase activity o) from a Phenyl-Sepharose column, wherein the elution was carried out by ammonium sulfate concentration gradient (30 to 0%) and
5 ethyleneglycol concentration gradient (0 to 50%) to obtain fractions Nos. 1 to 100, followed by 50% ethyleneglycol to obtain fraction Nos. 101 to 120.

Fig. 3 shows an elution profile of protein (A280 ●) and a megakaryocyte differentiation factor
10 (acetylcholine esterase activity o) from an S-Sepharose column, wherein the elution was carried out by NaCl concentration gradient (0 to 0.5M) to obtain fractions Nos. 1 to 100.

Fig. 4 shows an elution profile of protein
15 (A280   ) and megakaryocyte differentiation factor (acetylcholine esterase activity o) from a Hiroad 26/60 Superdex 75 Pg column, and a result of analysis of the resulting fractions by SDS-PAGE (lower part of the Figure).

20 Fig. 5 is an electrophoretic pattern showing a result of SDS-PAGE analysis of the purified megakaryocyte differentiation factor.

Fig. 6 shows a result of an iselectroric focusing of the purified megakaryocyte differentiation factor.

25 Fig. 7 is an electrophoretic pattern showing a result of sugar chain analysis for the purified megakaryocyte differentiation factor by SDS-PAGE, wherein the column 1 shows a result for a non-treated megakaryocyte differentiation factor, and the
30 column 2 shows a result for an endoglycosidase F-treated megakaryocyte differentiation factor (note, the band near

the 35 kD position is derived from the enzyme preparation).

Fig. 8 is a graph comparing acetylcholine esterase activity of megakaryocytes derived from mouse bone marrow cells cultured for 5 days in the presence or absence of purified megakaryocyte differentiation factor (55 kDa protein) and with or without addition of IL-6.

Fig. 9 shows a result of acetylcholine esterase staining of mouse bone marrow cells cultured for 4 days after the addition of IL-3, in the presence (B) or absence (A) of purified megakaryocyte differentiation factor.

Fig. 10 shows a result of May-Gruenwald-Giemsa's staining of mouse bone marrow cells cultured for 4 days after the addition of IL-3, in the presence (B) or absence (A) of purified megacaryocyte differentiation factor.

Fig. 11 shows detection of RNA with a DNA probe (PCR product amplified with KY100 and N1065 described in Example 2.1) wherein the RNA was extracted from cultured Bombyx mori cells infected with recombinant virus (TPO55-Bm NPV) comprising a gene coding for megakaryocyte differentiation factor (Lane B), cells infected with wild type virus (B6E) (Lane A) or from non-transformed cells (Lane C).

Fig. 12 is a graph showing an expression of megakaryocyte differentiation factor (TP55) in hemolymph of Bombyx mori after reparation by Matrex Blue A column chromatography.

DETAILED DESCRIPTION

As starting materials for isolating the present

megakaryocyte differentiation factor, human cells, such as human cancer cells, preferably human epidermoid carcinoma cells A431, particularly preferably human epidermoid carcinoma A431 grown in a protein-free medium can be mentioned.

In addition to the above-defined megakaryocyte differentiation factor, the present invention relates megakaryocyte differentiation factors obtainable from transformants such as cells or animals constructed by gene technology and having the same amino acid sequence as the above-defined megakaryocyte differentiation factor, an amino acid sequence wherein a portion of the above-defined megakaryocyte differentiation factor is deleted, an amino acid sequence wherein a portion of the above-defined megakaryocyte differentiation factor is replaced with other amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid sequence is added to the above-defined megakaryocyte differentiation factor, or having an amino acid sequence including a combination of said modifications.

Moreover, the present invention relates to megakaryocyte differentiation factor having the amino acid sequence shown in SEQ ID NO: 30, an amino acid sequence wherein a portion of the amino acid sequence shown in SEQ ID NO: 30 is deleted, an amino acid sequence wherein a portion of the amino acid sequence shown in SEQ ID NO: 30 is replaced with an other amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid sequence is added to the amino acid sequence shown in SEQ ID NO: 30, or having an amino acid sequence including a combination of said

modifications.

The present invention also relates to genes coding for the above-mentioned megakaryocyte differentiation factors. The present invention further relates a process for production of the megakaryocyte differentiation factors using the gene by means of gene recombination technology. The gene recombination technology follows conventional procedures by using a synthetic or natural polynucleotide coding for the amino acid sequence of the native megakaryocyte differentiation factor, an amino acid sequence wherein a portion of the native amino acid sequence is deleted, an amino acid sequence wherein a portion of the native amino acid sequence is replaced with other amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid is added to the native amino acid sequence, or coding for an amino acid sequence including a combination of said modifications, but not limited to the above.

The above-mentioned various modification can be carried out by a conventional technique such as site-specific a mutagenesis.

The number of amino acids involved in the modification such as addition, deletion or replacement is not limited, but as for addition the number of amino acid depends on the number of amino acids, for example, that of the functional peptide used in a hybrid protein with the megakaryocyte differentiation factor of the present invention or that of a signal peptide added to the present factor, namely depends on purpose of the modification, as for deletion the number of amino acids may be designed or determined so as to maintain

megakaryocyte differentiation activity and it is, for example, 1 to 30, preferably 1 to 20 or it can be that of region other than the active region of the present factor, and as for replacement the number of amino acids also may be designed or determined so as to maintain megakaryocyte differentiation activity and it is, for example, 1 to 10, preferably, 1 to 5.

An addition or improvement of an signal sequence, choice of host-vector system, and improvement of expression regulatory region may provide efficient expression. In addition, a host may be chosen to provide a glycosylated product. Moreover, a polynucleotide coding for at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9 may be used as a DNA probe for cloning a gene.

The present invention further provides a pharmaceutical composition comprising a megakaryocyte differentiation factor as an effective ingredient. The pharmaceutical composition is preferably used as a medicament for thrombocytopenia.

In addition, the present megakaryocyte differentiation factors can be used to obtain specific antibodies according to a known procedure.

Now, the present invention is explained in more detail.

(1) Starting material

As a starting material for obtaining a novel protein of the present invention, a culture supernatant of cells derived from human epidermoid carcinoma cell A431 (ATCC CRL 1555), rendered to be capable of growing in a protein-free medium according to Yamaguchi et al.

method (Yamaguchi N. et al., Cancer Res. 50, 7008, 1991),
is mentioned. This cell line was designated as Human
epidermoid carcinoma SBM 330, and deposited as
FERM BP-3911 with the Fermentation Research Institute
5   Agency of Industrial Science and Technology, 1-3, Higashi
1-chome, Tsukuba-shi Ibraki-ken (307) Japan, on July 1,
1992.

          (2)   Assay method for megakaryocyte differentiation
factor

10   To assay a megakaryocyte differentiation
factor, megakaryocyte-series cell lines (for example, CMK
cells or cells derived therefrom), or mouse bone marrow
cells may be used. For example, activity of
acetylcholine esterase which is known to be specifically
15   detected in murine megakaryocytes is carried out using
mouse bone marrow cells according to the Ishibashi et al.
method (Ishibashi, T. et al., Proc. Natl. Acad. Sci. USA
86, 5953, 1989). In addition, histochemical detection of
megakaryocytes is carried out by subjecting cultured bone
20   marrow cells to acetylcholine esterase staining and May-
Gruenwald-Gremsa's staining and the observing the shape
of the stained cells.

          (3)   Purification of megakaryocyte differentiation
factor

25   The megakaryocyte differentiation factor can be
purified, for example, starting from a culture
supernatant of A431 cells cultured in a protein-free
medium, concentration by ultrafiltration, and column
chromatography using, for example, Matrex Blue A
30   (Amicon), Q-Sepharose (Pharmacia), phenyl-Sepharose
(Pharmacia), S-Sepharose (Pharmacia) and Hiload 26/60

Superdex 75 (Pharmacia) alone or in combination. Protein is monitored by measuring A280 nm.

(4) Determination of partial amino acid sequence of megakaryocyte differentiation factor

5   To determine an amino acid sequence, the megakaryocyte differentiation factor purified in the section (3) is digested into fragments with a protease such as Achromobacter Protease I (API) at 37°C for 2 hours. The resulting peptide fragments are separated 10 and recovered by reverse phase HPLC (acetonitrile gradient in 0.1% trifluoroacetic acid) using YMC-Pack AM-303 column. The peptide fragments thus obtained are subjected to a sequencer such as a gas-phase sequencer obtained from, for example, Applied Biosystem. A 15 definite purification process and detailed properties of the megakaryocyte differentiation factor are described in Example 1.

The present invention also provides gene coding for megakaryocyte differentiation factor. The gene may be 20 cDNA prepared from mRNA, genomic DNA, and synthetic DNA. For example, cDNA can be cloned by a polymerase chain reaction (PCR) using DNA (nucleotide) primers designed on the basis of partial amino acid sequences as shown in Example 1 of a megakaryocyte differentiation factor 25 purified from the above-mentioned human cells, such as human epidermoid carcinoma cells, for example A431 cells. The cloning is described in detail in Examples 1 and 2.

The gene of the present invention further includes DNAs coding for protein or glycoprotein having 30 megakaryocyte differentiation activity and hybridizing with the nucleotide sequence of SEQ ID NO: 30.

Nucleotide sequence of DNA cloned in Example 2 and an amino acid sequence predicted from the nucleotide sequence are shown in SEQ ID NO: 30.

Thus once an amino acid sequence is determined,
5   various mutated megakaryocyte differentiation factors, such as a polypeptide, wherein one or more than one amino acid is added to the native amino acid sequence or the amino acid sequence shown in SEQ ID NO: 30 and maintaining megakaryocyte differentiation factor
10   activity, a polypeptide wherein one or more than one amino acid is deleted from the native amino acid sequence or the amino acid sequence shown in SEQ ID NO: 30 and maintaining megakaryocyte differentiation factor activity, a polypeptide wherein one ore more than one
15   amino acid is replaced with one or more than one other amino acid, in the native amino acid sequecne or the amino acid sequence shown in SEQ ID NO: 30, and maintaining megakaryocyte differentiation factor activity, or a polypeptide including a combination of the
20   above-mentioned modifications such as addition, deletion and/or replacement of amino acids maintaining megakaryocyte differentiation factor activity can be designed and produced.

According to the present invention, although the
25   nucleotide sequence shown in SEQ ID NO: 30 is disclosed, gene coding for the present megakaryocyte differentiation factor is not limited thereto. Once an amino acid sequence of the present native megakaryocyte differentiation factor or an amino acid sequence of a
30   mutated megakaryocyte differentiation factor is determined, according to the degeneracy of genetic code,

various nucleotide sequences coding for the same amino acid sequence can be designed and prepared. In this case, codons used with a high frequency in a chosen host are preferably used.

5   A gene coding for the present megakaryocyte differentiation factor can be obtained as cDNA according to Example 2, but the gene is not limited to cDNA. Namely, once a nucleotide sequence coding for an amino acid sequence of native megakaryocyte differentiation

10   factor is determined, a gene coding for the native megakaryocyte differentiation factor can be cloned as a cDNA according to a strategy different from the strategy definitely described herein, and moreover a desired gene can be cloned from the genome of a cell producing the

15   megakaryocyte differentiation factor.

Where a desired gene is cloned from the genome, various primer nucleotides or probe nucleotides used in Example 2 can be used as probes for screening genomic DNA fragments. Moreover, other probes designed on the basis

20   of the nucleotide sequence described in SEQ ID NO: 30 can be used. General procedures for cloning a desired DNA from a genome are well known in the art (see Current Protocols In Molecular Biology, John Wiley & Sons, Chapters 5 and 6).

25   Gene coding for the native megakaryocyte differentiation factor of the present invention can also be prepared by chemical synthesis. It is easy in the art to chemically synthesize DNA using an automated DNA synthesizer, for example, Applied Biosystems 396 DNA/RNA

30   synthesizer. Accordingly a person with ordinary skill in the art can easily synthesize DNA having the nucleotide

sequence shown in SEQ ID NO: 30.

A gene coding for the present native megakaryocyte differentiation factor using codons different from native codons, and a gene coding for a mutated megacaryocyte differentiation factor can be prepared by chemical synthesis as described above. Alternatively they can be obtained by site-specific mutagenesis using as a templete a DNA or RNA having a nucleotide sequence shown in SEQ ID NO: 30 together with mutagenic primers (for example, see, Current Protocols In Molecular Biology, John Wiley & Sons, Chapter 8).

Once a gene coding for the present megakaryocyte differentiation factor is obtained, the gene can be used to produce a recombinant megakaryocyte differentiation factor according to a conventional gene recombination technology. Namely, a DNA coding for the present megakaryocyte differentiation factor is inserted into an appropriate expression vector, the vector is introduced to appropriate host cells, the transformed host cells are cultured, and the target megakaryocyte differentiation factor is recovered from the culture (cells or medium). The present megakaryocyte differentiation factor can be biochemically or chemically modified, for example, N-terminal acylated.

In addition, on the basis of the nucleotide sequence shown in SEQ ID NO: 30, a protein data base was searched with fasta Program (GCG package). As a result, the megakaryocyte differentiation factor belongs to the super family of serine protease inhibitor. On the other hand, in human leucocyte elastase inhibitor, chicken ovalbumin Y gene product, human plasminagen activator inhibitor 2

and human squamous cell carcinoma antigen, which are
similar to the present megakaryocyte differentiation
factor in their expected steric structure, and
distribution of hydrophobic and hydrophilic amino acids,
the N-terminal portion is not cleaved and forms a signal
peptide. Accordingly, there is a possibility that in the
present megakaryocyte differentiation factor, the N-
terminal portion may function as a signal peptide and the
megakaryocyte differentiation factor may be secreted
without cleavage of the signal peptide. Further, the
present megakaryocyte differentiation factor may be
modified so that the first methionine is deleted and the
second alanine is acetylated.

As hosts, both the prokaryote and enkaryote can be
used. As prokaryote, bacteria such as <u>Escherichia</u> <u>coli</u>
the genus <u>Bacillus</u>, for example, <u>B.</u> <u>subtilis</u> and the like
can be used. As eukaryote, yeast such as the genus
<u>Saccharomyces</u>, for example, <u>S.</u> <u>serevisiae</u>, insect cells
such as <u>Spodoptera</u> <u>frugiperda</u> cells, <u>Cabbage</u> <u>looper</u>
cells, <u>Bombyx</u> <u>mori</u> cells, animal cells such as human
cells, monkey cells, mouse cells and the like can be
used. Moreover, insects per se, such as <u>Bombyx</u> <u>mori</u> may
be used.

As expression vectors, plasmid, phage, phagemid,
virus such as bacuro virus, vaccinia virus or the like
can be used. A promoter in an expression vector is
selected depending on host used. For example, lac
promoter, trp promoter and the like can be used as
bacterial promoters, and adhl promoter, pqk promoter and
the like can be used as yeast promoters. On the other
hand, baccuro virus polyhedrin promoter can be used as

insect promoter, and Simian virus 40 early or late
promoter can be used for animal cells.

Transformation of a host with an expression vector
can be carried out according to conventional procedures
5   well known in the art, and these procedures are described
in, for example, Current Protocols in Molecular Biology,
John Wiley & Sons. Culturing of a transformant also can
be carried out according to a conventional procedure.

Purification of a megakaryocyte differentiation
10   factor from a culture or an insect body can be carried
out according to conventional procedures used for
isolation and purification of a protein, for example,
ultrafiltration, various types of column chromatography
such as Q-Sepharose column chromatography and the like.

15   EXAMPLES

Next, the present invention is further explained by
Examples.

Example 1.   Purification of megakaryocyte
differentiation factor

20   (1)   Culturing of A431 cells

Frozen SBM 330 cells conditioned in a protein-
free medium from A431 cells were thawed, and cultured in
a primary medium (Ham's F12 medium containing 10% fetal
bovine serum). Namely, the cells were plated in 10 T
25   flasks having a culture area of 150 cm^2, and cultured to
confluence at 37°C in the presence of 5% CO_2. Next, the
cells were peeled off with a 0.25% trypsin solution
(Chiba Kessei) and subcultured in 10 roller bottles
having a culture area of 850 cm^2 at 37°C and 0.5 rpm for
30   about 3 days to recover 1.8 x 10^9 cells. The cultured
cells were attached to a ceramic core (S-451) of an Opti-

cell incubator (Charles River Inc. Wilmington, MA) and perfusion culturing was started using 10L of a primary medium.

5   The perfusion culture was carried out at 37°C with supplying oxygen at 150 mmHg. The primary medium was replaced with a protein-free medium as follow. Namely, after culturing for about 7 days in a primary medium, a protein-free medium was supplied to the culture at a rate of 20L/day, and simultaneously the culture
10   supernatant was recovered from the culture at the same rate. As a result, the primary medium containing serum was substantially completely replaced with the protein-free medium by supplying about 100L of the protein-free medium. Thereafter, cell culture supernatant was
15   continuously recovered to obtain 1000L of cell culture supernatant. A part (about 300L) of the cell culture supernatant thus obtained was concentrated to 2L using an ultrafiltration membrane (Milipore, Bedford, MA; MW 10,000 cut) and the concentrate was dialyzed against
20   20 mM Tris/HCl buffer (pH 7.4).

(2)   Assay of megakaryocyte differentiation factor using mouse bone marrow cells

Myeloid cells were pushed out of the femur of female BDF_1 mouse and suspended in an α-MEM medium (Flow
25   Laboratories, Inc. McLean, VA, USA). Percoll layers having different densities (Pharmacia LKB Biotechnology, Tokyo) were overlaied, and the bone marrow cell suspension was put thereon, followed by centrifugation at 400 x g for 20 minutes. Mononuclear cells collected at
30   the interface of a layer having a density of 1.07 g/ml and a layer having a density of 1.08 g/ml were recovered

and washed once with α-MEM containing 10% FBS, and resuspended in the same medium containing 0.5 mM diisopropylfluorophosphate. The suspension was then put into a plastic cell culture dish (Corning, NY, USA) and cultured at 37°C in 5% carbon dioxide and 95% air for 2 hours. During the culturing, at one hour from the start of culturing, the cell culture dish was replaced with a new one. After the culturing, cells were washed with 10% FBS/α-MEM three times.

The non-adherent bone marrow mononuclear cells thus obtained were suspended in 10% FBS/1% BSA/0.1 mM 2-mercaptoethanol/α-MEM, and plated in a 96-well microplate (Corning) in an amount of 5×10^4 cell/well. If necessary, to a test sample were added 25 U/ml mouse recombinant IL-3 (Genzyme Corporation, Cambridge, MA, USA) and 1 to 2 µg/ml anti-IL-6 antibody (Boehringer Mannheim, Mannheim, FRG). Where anti-IL-6 antibody was added, the test sample and the antibody were preincubated at 37°C for an hour prior to seeding the cells.

The culturing was carried out at 37°C in 5% CO_2 - 5% O_2 - 90% N_2 for 4 to 5 days. After culturing cells in each well of the microplate they were washed twice with PBS, and lysed with 180 µl of 0.2% (w/v) Triton X-100, 1 mM EDTA, 0.12M NaCl, 50 mM HEPES (pH 7.5), and 20 µl of a substrate, 5.6 mM acetythiolcholine iodide, was added thereon. After culturing with shaking at a room temperature for an hour, 20 µl of the solution was transferred to a microplate for fluorescent assay (Dynatech Micro FLUOR "B" Plate).

To the microplate were added 20 µl of 0.4 mM

7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarine
in acetonitrile and 160 μl of 0.2% (w/v) Triton X-100,
1 mM EDTA, 50 mM sodium acetate (pH 5.0), and fluorescent
emission was measured by a fluorometer (excitation

5   365 nm, emission 450 nm).

For acetylcholine esterase staining, the cells
were centrifuged by a Cytospin to adhere the cells onto a
slide glass, and fixed with 5% glutaraldehyde, 10 mM
phosphate buffer (pH 6.7) for 15 minutes, and as a

10   substrate acetylthiocholine was used according to
Mizoguchi's method (Method for Culturing Hemopoietic Stem
Cells, Chugai Igaku, 1986, ed. Y. Miura, pp82-88).
Namely, after fixing the cells, the slide glass was
washed with 0.1M phosphate buffer (pH 6.0), and to each

15   slide glass was layered a mixture of 1.5 ml of 0.67 mg/ml
acetylthiocholine iodide, 0.1M phosphate buffer (pH 6.0),
0.2 ml of 30 mM $CuSO_4$, 0.2 ml of 5 mM potassium
ferricyanide and 0.1 ml of 0.1M sodium citrate. The
slide glass was incubated at a room temperature for

20   4 hours, and washed with water. May-Gruenwald-Giemsa's
staining is well known in hematology and was carried out
using reagents available from E. Merck (Darmstadt, FRG)
wherein May-Gruenwald's staining was carried out for
4 minutes and Giemsa's staining was carried out for

25   10 minutes.

(3)   Purification of megakaryocyte differentiation
factor

A culture supernatant concentrate of A431 cells
was dialyzed and centrifuged to obtain a supernatant

30   which was then applied to a Matrex Blue A Column
equilibrated with 20 mM Tris/HCl buffer (pH 7.4), and

after washing the column with the same buffer, a bound
fraction was eluted with the same buffer containing 2M
NaCl. Megakaryocyte differentiation activity detected by
the above-mentioned method was found in the bound
fraction. Therefore, the bound fraction was dialyzed
against 20 mM Tris/HCl buffer (pH 7.4) and applied to a
Q-Sepharose column equilibrated with the same buffer.
The column was thoroughly washed and a megakaryocyte
differentiation factor was eluted by NaCl gradient (see
Fig. 1). This factor was eluted near the position of 0.3
to 0.5M NaCl.

The active fraction obtained from Q-Sepharose
was made ammonium sulfate 30% saturation, and was applied
to a phenyl Sepharose column equilibrated with 20 mM
Tris/HCl buffer (pH 7.4) containing 30% saturation of
ammonium sulfate. The megakaryocyte differentiation
factor was eluted by simultaneously forming concentration
gradients of ammonium sulfate (30% to 0%) and
ethyleneglycol (0 to 50%) (see Fig. 2). The
megakaryocyte differentiation factor measured in the
presence of anti-IL-6 antibody was observed over several
fractions in the beginning of the concentration gradient
formation.

The fractions thus obtained were combined,
thoroughly dialyzed against 50 mM MES/NaOH buffer
(pH 6.0), and applied to an S-Sepharose column
equilibrated with the same buffer. A bound fraction was
eluted by a 0 to 0.5M NaCl concentration gradient (see
Fig. 3). Although activity was widely distributed,
relatively high activity was found in the beginning of
the concentration gradient formation. The fractions

obtained from S-Sepharose were applied to a Hilord 26/60 Super Dex 75 (Pharmacia) column for gel filtration. The column used had be previously equilibrated with the same buffer, and elution was carried out with the same buffer (see Fig. 4). Activity of megakaryocyte differentiation activity was eluted near the position corresponding to a molecular weight of 55 to 57 kDa.

According to the above-mentioned steps, about 80 μg of a fraction showing two bands near 55 to 57 kDa as analyzed by SDS-PAGE was obtained from 300L of a culture supernatant of A431 derived cells (see Fig. 5). The two bands were correlated with the activity (Fig. 1). Accordingly, it was concluded that the two bands observed in said fraction correspond to a desired megakaryocyte differentiation factor.

(4)   Properties of megakaryocyte differentiation factor

The present megakaryocyte differentiation factor has the following properties.

1)   Molecular weight: about 55 kDa (gel filtration and SDS-PAGE) (Figs. 4 and 5)

The present factor exhibits two bands in SDS-PAGE, and there is no difference in mobility between reducing condition and a non-reducing condition. Therefore the factor does not have intermolecular disulfide linkage.

2)   Isoelectric point: 6.5 ± 0.5 (Fig. 6)

A several bands are observed in the above-mentioned range.

3)   The above-mentioned heterogeneity of the present factor can be explained as heterogeneity in a

sugar chain structure of glycoprotein. Namely, where the present factor is treated with endoglycosidase F which is an asparagine linked sugar removing enzyme, the molecular weight of the present factor decreased to a molecular weight of about 40 kDa, and the heterogeneity also decreased (Fig. 7), in SDS-PAGE. In addition, where a fraction exhibiting a single band and a fraction exhibiting two bands were digested with API and a peptide map was prepared by fractionation by a reversed phase HPLC, the difference was not observed between the two fractions.

4)   The present factor contains at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9.

5)   Biological activity

Where mouse bone marrow cells were cultured in the presence of a purified megakaryocyte differentiation factor and IL-3, the proliferation and differentiation of megakaryocytes are observed (Figs. 8, 9 and 10). Fig. 8 shows the result of measurement for acetylcholine esterase activity of megakaryocytes; Fig. 9 shows the result of acetylcholine esterase staining of the cultured cells (× 20); and Fig. 10 shows the result of May-Gruenwald-Giemsa's staining of the cultured cells (× 100). In both the Figs. 9 and 10, it is seen that megakaryocytes increased in the presence of a megakaryocyte differentiation factor (B) in comparison with in the absence of the same (A).

(5)   Structure of megakaryocyte differentiation factor

To characterize the structure of a purified megakaryocyte differentiation factor, the factor

was digested with API and the structures of the resulting fragments were determined. After the digestion of the factor with API, each fragment was recovered by reversed phase HPLC, and the structures for appropriate fractions were determined. As a result, the peptide fragments had amino acid sequences shown in SEQ ID NO: 1 to 9.

Example 2. Determination of Structure of CDNA for megakaryocyte differentiation factor

1. Analysis of cDNA nucleotide sequence of megakaryocyte differentiation factor by PCR (1)

Oligonucleotides NI065 (SEQ ID NO: 10; corresponding to 449-486 of SEQ ID NO: 30) and NI067 (SEQ ID NO: 11; corresponding to 1049-1080 of SEQ ID NO: 30) were synthesized by designing nucleotide sequences on the basis of the amino acid sequences shown in SEQ ID NO: 3 and 4 respectively.

Total RNA was purified from A431 cells using ISOGEN (Wako Pure Chemical) according to the manufacturer's instructions. RNA having poly A was purified from the total RNA, and a reaction was carried out using a 3'-RACE Kit (Gibco BRL). Namely, the above-mentioned oligomer NI065 and oligomer 3'-RACE adaptor primer (SEQ ID NO: 12) attached to the 3'-RACE Kit (Gibco BRL) were used to carry out polymerase chain reaction (PCR) according to instructions included with the kit.

The reaction product was then subjected to second PCR using the primer NI067 and the oligomer 3'-RACE adapter primer included in the 3'-RACE Kit (Gibco BRL) to obtain a DNA fragment of about 900 base pairs. Next, using a direct nucleotide sequence determination method for a PCR product, according to U. Gyllensten et.

al., Proc. Natl. Acad. Sci. USA 85: 7652 (1988), the DNA fragment of about 900 base pairs was directly used as a reaction substrate to determine a nucleotide sequence of a portion representing protein and a portion downstream of the protein portion using a Taqu Dye Deoxy Terminator Cycle Sequencing kit available from Applied Biosystem and a fluorescent nucleotide sequencer (Applied Biosystem, Type 370A) according to a manufacturers instruction. As a result, a sequence from nucleotide number 1081 to 1950 of SEQ ID NO: 30 was shown.

On the basis of this sequence, oligomer KY100 (SEQ ID NO: 13; corresponding to 1255-1236 of SEQ ID NO: 30) was synthesized. The reaction product obtained by the PCR using NI065 and the oligomer 3'-RACE adapter primer attached to the 3'-RACE Kit (Gibco BRL) was used as a reaction substrate to carry out a further PCR using NI065 and KY100. As a result, a DNA fragment of 807 base paires was obtained.

This DNA fragment of 807 base paires was directly used as a reaction substrate to determine its nucleotide sequence using Taq Dye Deoxy Terminator Cycle Sequencing kit available from Applied Biosystem and a fluorescent nucleotide sequencer according to a manufactures instruction. As a result, a nucleotide sequence from nucleotide number 487 to 1080 of SEQ ID NO: 30 was shown. On the basis of this sequence, oligomers NI073 (SEQ ID NO: 14; corresponding to 864-886 of SEQ ID NO: 30), NI074 (SEQ ID NO: 15; corresponding to 1012 to 992 of SEQ ID NO: 30), and NI075 (SEQ ID No: 16; corresponding to 802-782 of SEQ ID NO: 30) were synthesized.

2.   Analysis of cDNA nucleotide sequence of
megakaryocyte differentiation factor by PCR (2)

A.   Preparation of mRNA from megakaryocyte
differentiation factor expressing cell line (A431)

From 1.1g of frozen cells of human
epidermoid carcinoma cell line (A431), 25 μg of mRNA was
extracted and purified using an RNA extraction kit and an
mRNA purification kit available from Pharmacia-LKB.

B.   Preparation of DNA phage library from
megakaryocyte differentiation factor expressing cell line
(A431)

(1)   Synthesis of cDNA

From 5 μg of the mRNA derived from
A431, cDNA was synthesized using a cDNA synthesis kit
Time Saver available from Pharmacia-LKB.   First 5 μg of
mRNA dissolved in 20 μl of distilled water treated with
diethylpyrocarbonate (DEPC) was heated at 65°C for
10 minutes and cooled on ice.   11 μl of a first strand
reaction mixture, 1 μl of DTT solution and 1 μl of
130 U/ml NotI/oligomer 18 primer solution (Pharmacia-LKB)
were added thereto, and the mixture was incubated at 37°C
for an hour.

The reaction mixture was added to a
second strand reaction mixture, and the mixture was
incubated at 12°C for 30 minutes and 22°C for an hour,
and heated at 65°C for 10 minutes.   100 μl of a mixture
of phenol/chloroform/isoanylalcahol (25:24:1; abbreviated
as PC hereinafter) was added thereto, and the mixture was
vigorously stired, centrifuged at 14,000 × 9 for one
minute to obtain a supernatant, which was then

fractionated by a Sephacryl S-400 spin column
(Pharmacia LKB) to obtain 100 µl of cDNA solution.

(2)   Addition of EcoRI adaptor

To 100 µl of the cDNA solution were

5   added 5 µl of 10 U/ml EcoRI adaptor (Pharmacia LKB),
30 µl of polyethylene glycol buffer, 1 µl of 1/5 diluted
ATP solution and 1 µl of T4 DNA ligase, and the mixture
was incubated at 37°C for an hour. After heating at 65°C
for 10 minutes, 1.5 µl of ATP solution and 1 µl of T4

10   polynucleotide kinase were added thereto and the mixture
was incubated at 37°C for 30 minutes. After heating at
65°C for 10 minutes, 2 µl of 20 U/µl NotI was added to
the mixture, which was then incubated at 37°C for an
hour. 150 µl of PC was added to the mixture, which was

15   then vigorously stirred and centrifuged at 14,000 × g for
a minute, and the supernatant was fractionated on
Sephadex S-400 spin column to obtain 150 µl of cDNA
solution.

(3)   Incorporation of cDNA into phage

20   vector and in vitro packaging

After digesting with EcoRI and NotI,
to 15 µl of the cDNA solution were added 2 µg of
dephosphorylated λgt11D (Pharmacia LKB). After ethanol
precipitation, the precipitate was dissolved in 8 µl of

25   ligase buffer solution, and 1 µl of 1/75-diluted ATP
solution and 1 µl of T4 DNA ligase were added to the
solution, which was then incubated at 16°C for 30 minutes
and stored on ice.

An in vitro packaging reaction was

carried out using Gigapack II Gold (Stratagene), and a library of 3.22×10^6 pfu of recombinant phages was obtained from said 3 ligase reaction products. Said library was amplified in an <u>E. coli</u> Y1090 r⁻ host to

5   obtain 6.0×10^{10} pfu/ml of an A431 phage library stock.

          C.   Identification and isolation of cDNA fragment for megakaryocyte differentiation factor by PCR

          (1)   Amplification of cDNA insert fragment in A431 phage library by PCR

10   10 µl of a stock solution of 6.0×10^{10} pfu/ml A431 phage library (corresponding to 6.0×10^8 pfu) was used as a template DNA for PCR reaction, and 5 µl of 10 × PCR buffer, 8 µl of 1.25 mM 4 dNTPs, 2 µl of 1 OD/ml λgt11-forward primer (λgt 11F)

15   (SEQ ID NO: 17), 2 µl of 1 OD/ml λgt11-reverse primer (λgt 11R) (SEQ ID NO: 18), and 1 µl of 5 U/µl Taq DNA polymerase (Perkin Elmer Cetus) were added, and the total volume of the mixture was made to be 50 µl with DEPC-treated distilled water. 30 reaction cycles of 93°C for

20   a minute, 55°C for 2 minutes and 72°C for 3 minutes were carried out, and the reaction mixture was incubated at 72°C for 10 minutes. As a result of an analysis by 1% Agarose gel electrophoresis, a smeary pattern ranging 0.8 to 6 kb was shown.

25   (2)   PCR analysis using cDNA insert DNA amplification fragment mixture as a template and using TP7 (SEQ ID NO: 20; corresponding to 683-703 of SEQ ID NO: 30), /TP10, TP7/TP6 (SEQ ID NO: 19; corresponding to 1036-1001 of SEQ ID NO: 30), TP8 (SEQ ID NO: 21;

30   corresponding to 941-964 of SEQ ID NO: 30), TP10 (SEQ ID

NO: 22; corresponding to 1036-986 of SEQ ID NO: 30) and TP8/TP6 as primers

1 µl of a 1/5000-diluted solution of the above-mentioned PCR reaction product was used as a template DNA for PCR reaction, and 5 µl of 10 × PCR buffer, 8 µl of 1.25 mM 4 dNTPs, 2 µl each of 1 OD/ml primers in combination as described hereinafter and 1 µl of Perfect Match (Stratagene) were added, and a total volume of the mixture was made to 49 µl with DEPC-treated distilled water.

The reaction mixture was heated at 95°C for 5 minutes and 60°C for 5 minutes, 1 µl of 5 U/µl Taq DNA polymerase (Perkin Elmer Cetus) was added thereon, and 30 cycles of 94°C for a minute, 60°C for 2 minutes and 72°C for 3 minutes were carried out, followed by an incubation at 72°C for 10 minutes. As primers, TP7/TP10, TP7/TP6, TP8/TP10, and TP8/TP6 were used.

As a result of an analysis of the PCR reaction product by 2% agarose gel electrophoresis, bands of 354 bp, 354 bp, 96 bp and 96 bp were obtained corresponding to primers respectively.

(3) Analysis for primary sequence of PCR amplification products (354 bp) obtained by using primers TP7/TP10 and TP7/TP6

The bands of said PCR amplification products (354 bp) obtained using primers TP7/TP10 and TP7/TP6 were excised from the 2% agarose gel after the electrophoresis, and to the excised agarose gel pieces was added 50 µl of DEPC-treated distilled water, and the

mixture was heated at 45°C for 30 minutes. To 2 μl of
this solution as a template DNA, were added 5 μl of
10 × PCR buffer, 8 μl of 1.25 mM 4 dNTPs, 2 μl each of
1 OD/ml primers in combination and 1 μl of Perfect Match
5   (Stratagene), and a total volume of the reaction mixture
was made to 49 μl with DEPC-treated distilled water. The
mixture was heated at 95°C for 5 minutes and 60°C for
5 minutes, 1 μl of 5 U/μl Taq DNA polymerase (Perkin
Elmer Cetus) was added, and 30 reaction cycles of 94°C
10   for a minutes, 60°C for 2 minutes and 72°C 3 minutes were
carried out, followed by incubation at 72°C for
10 minutes. As primes for the above-mentioned PCR
reaction, TP7/TP10 and TP7/TP6 were used. Bands of the
PCR reaction products (each 354 bp) were excised from 2%
15   agarose gel of the electrophoresis, extracted and
purified, and the product was inserted into pCR II
(Invitrogen), which was then used to transform E. coli
INVαF' (Invitrogen). Plasmid DNA was extracted and
purified, and it was confirmed by EcoRI digestion that
20   DNA fragment of 354 bp had been inserted.

Primary sequence of the DNA insert
fragment was analyzed using M13 forward primer (M13F)
(SEQ ID NO: 23) and M13 reverse primer (M13R) (SEQ ID
NO: 24) (Aplied Biosystem's automated sequencer,
25   Model 370A). As a result, a 296 bp sequence
corresponding to the nucleotide number 704 to 999 of SEQ
ID NO: 30 was found, and this sequence contained C-
terminal 3 amino acids (XRK; but ERK from DNA nucleotide
sequence) of SEQ ID NO: 9 corresponding to the downstream
30   portion of primer TP7, N-terminal 5 amino acids (ADLSG)

of SEQ ID NO: 6 corresponding to the upstream portion of primer TP6, and 8 amino acids (YLRALGLK) of SEQ ID NO: 5 corresponding to primer TP8, revealing that the PCR reaction products (each 354 bp) is a part of cDNA coding for megakaryocyte differentiation factor.

3.   Screening of cDNA coding for megakaryocyte differentiation factor

A.   Preparation of cDNA plasmid library from megakaryocyte differentiation factor expressing cell line (A431)

(1)   Synthesis of first strand cDNA

From 5 μg of mRNA derived from A431 cell line, cDNA was synthesized using a Super Script plasmid system available from GIBCO. First 2 μl of NotI primer adaptor was added to 5 μg of mRNA dissolved in 5 μl of diethylpyrocarbonate (DEPC)-treated distilled water, and the mixture was heated at 70°C for 10 minutes and cooled on ice. 4 μl of 5 × first strand buffer, 2 μl of 0.1 M DTT solution, 1 μl of 10 mM 4dNTPs and 1 μl of DEPC-treated distilled water were added thereto, and the mixture was incubated at 37°C for 2 minutes. 5 μl of a Super Script reverse transcriptase was added to the reaction mixture, which was then incubated at 37°C for one hour and then put on ice to stop the reaction.

(2)   Synthesis of second strand cDNA

To 18 μl of 20 μl reaction mixture for the first strand cDNA synthesis were added 93 μl of DEPC-treated distilled water, 30 μl of 5 × second strand buffer, 3 μl of 10 mM 4 NTPs, 1 μl of 10 U/μl E. coli DNA ligase, 4 μl of 10 U/μl E. coli DNA polymerase and 1 μl

of 2 U/μl <u>E. coli</u> RNase H, and the mixture was incubated at 16°C for 2 hours. 2 μl (10 U) of T4 DNA polymerase was added to the reaction mixture, which was then incubated at 16°C for 5 minutes.

5   The reaction mixture was put on ice, and after adding 10 μl of 0.5M EDTA and 150 μl of PC thereto, was vigorously stirred and centrifuged at 14,000 × g for 10 minutes, and 140 μl of the supernatant was transferred to a fresh centrifuge tube. 70 μl of 7.5M ammonium acetate and 0.5 ml of ethanol were added to the supernatant, which was then allowed to stand at -80°C for 30 minutes. The mixture was centrifuged at 14,000 × g for 10 minutes, and after removing the supernatant, the precipitate was washed with 0.5 ml of 70% ethanol and dried under a reduced pressure.

                    (3)   Addition of BstXI adapter

                    The above-mentioned cDNA precipitate was dissolved in 25 μl of DEPC-treated distilled water, 10 μl of 5 × T4 DNA ligase buffer, 10 μl of BstXI adapter (Invitrogen) and 5 μl of T4 DNA ligase were added to the solution, which were then incubated at 16°C for 16 hours. 50 μl of PC was added to the mixture, which was the vigorously stirred, and centrifuged at 14,000 × g for 5 minutes. 45 μl of the supernatant was transferred to a fresh centrifuge tube. 25 μl of 7.5M ammonium acetate and 150 μl of ethanol were added to the tube, which was stirred and allowed to stand at -80°C for 30 minutes. After centrifuging at 14,000 × g for 10 minutes to remove supernatant, the precipitate was washed with 0.5 ml of

70% ethanol and dried under a reduced pressure.

(4) NotI digestion

The above-mentioned cDNA precipitate was dissolved in 41 µl of DEPC-treated distilled water, and 5 µl of REAct 7 buffer and 4 µl of NotI were added to the solution, which was incubated at 37°C for 2 hours. 50 µl of PC was added to the mixture, which was then vigorously stirred and centrifuged at 14,000 × g for 10 minutes, and 45 µl of the supernatant was transferred into a centrifuge tube.

(5) Elimination of adapter and size fractionation of partial cDNA

The above-mentioned cDNA solution was fractionated using a Quick Spin Column Linker 5 (Boehringer Mannheim). 50 µl of 40 µg/µl cDNA was obtained.

(6) Incorporation of cDNA into phagevector and transformation of E. coli

To 37.5 µl of the above-mentioned cDNA solution were added 12.5 µl of pCR/CMV (Invitrogen) vector (29 µg/µl) digested with Not I and BstXI, and further added were 400 µl of Takara Ligation kit A solution and 50 µl of B solution, and the mixture was incubated at 16°C for 30 minutes, and 1 ml of Max Efficiency DH5α competent cells (BRL) were transformed to obtain 71,550 recombinant clones. All colonies were collected from plate (2.86 × 10⁷ cells/ml) and stored at -80°C in the presence of 20% glycerol.

B. Screening of megakaryocyte differentiation factor cDNA by colony hybridization

Using the cDNA plasmid library derived
from A431 cell line, a total of 227,000 (3700/plate)
colonies were formed on 60 plates of 9 cm diameter, and
the colonies were replicated to nitrocellulose filters.

5 A probe was prepared by carrying out PCR (as described
hereinbefore) using primer NI 067 and 3'-RACE adaptor
(GIBCO BRL) to obtain a 900 bp PCR product, digesting the
PCR product with Bam HI to obtain two DNA fragments
(0.5 kb and 0.4 kb) and nick-translating the DNA

10 fragments with [α-^{32}P]dCTP.

For the colony hybridization, the filter
was incubated in 5 × SSC, 25 mM phosphate buffer
(pH 7.4), 5 × Denhaldt's solution, 1% SDS, 100 µg/ml heat
denatured salmon sperm DNA and 50% formamide at 42°C for

15 18 hours, and washed with 5 × SSC, 0.1% SDS at 40°C for
20 minutes and 45°C for 20 minutes. Detection was
carried out by exposing BAS 2000 (Fuji Film) for
18 hours.

First, second and third screening was

20 carried out to obtain 4 clones, i.e., TP290, TP308, TP310
and TP317. The length of insert cDNA was 1.2 kb, 1.1 kb,
1.2 kb and 1.2 kb respectively. The TP290, TP310 and
TP317 cover a region downstream from the nucleotide
number 685 of SEQ ID NO: 30.

25 **4. Analysis of cDNA nucleotide sequence coding for
megakaryocyte differentiation factor by PCR (3)**

A. Preparation of mRNA from megakaryocyte
differentiation factor expressing cell line (HPC-Y11)

From 1.1g of frozen cells of human

30 pancreatic cancer cell line (HPC-Y11), 50 µg of mRNA was
extracted and purified using an RNA extraction kit and

mRNA purification kit available from Pharmacia-LKB.

B.   Preparation of cDNA phage library from megakaryocyte differentiation factor expressing cell line (HPC-Y11)

(1)   Synthesis of cDNA

From 5 µg of mRNA derived from HPC-Y11, cDNA was synthesized using a Pharmacia-LKB's Time Saver cDNA synthesis kit. First, 5 µg of mRNA was dissolved 20 µl of diethylpyrocarbonate (DEPC)-treated distilled water, and the solution was heated at 65°C for 10 minutes and cooled on ice. 11 µl of first strand reaction mixture, 1 µl of DTT solution and 1 µl of NotI/oligomer 18 primer solution were added to the mixture, which was then incubated at 37°C for an hour.

100 µl of second strand reaction mixture was added to the mixture, which was then incubated at 12°C for 30 minutes and at 22°C for an hour, and heated at 65°C for 10 minutes. 100 µl of phenol-chloroform-isoamyl alcohol (25:24:1, abbreviated as PC) was added to the mixture, which was then vigorously stirred and centrifuged at 14,000 × g for a minute, and the supernatant was fractionated using a Sephacryl S-400 spin column (Pharmacia-LKB) to obtain 100 µl of cDNA solution.

(2)   Addition of EcoRI adaptor

To 100 µl of the cDNA solution, were added 5 µl of EcoRI adapter (Pharmacia-LKB), 30 µl of polyethylene glycol, 1 µl of ATP solution and 1 µl of T4 DNA ligase, and the mixture was incubated at 37°C for an hour. After heating at 65°C for 10 minutes, 1.5 µl of

ATP solution and 1 µl of T4 polynucleotide kinase were added to the mixture, which was then incubated at 37°C for 30 minutes. After heating at 65°C for 10 minutes, 12 µl of Not I was added to the mixture, which was then

5   incubated at 37°C for an hour. 150 µl of PC was added to the mixture, which was then vigorously stired and centrifuged at 14,000 × g for a minute, and the supernatant was fractionated using a Sephacryl S-400 spin column to obtain 150 µl of cDNA solution.

10   (3)   Incorporation of cDNA into phage vector and in vitro packaging

To 15 µl of the cDNA solution, was added 2 µg of λgt 11D (Pharmacia-LKB) which had been digested with EcoRI and Not I and dephosphorydated, and

15   after ethanol precipitation, the precipitate was dissolved in 8 µl of ligase buffer. 1 µl of 1/75-diluted ATP solution and 1 µl of T4 DNA ligase were added to the mixture, which was then incubated at 16°C for 30 minutes and stored on ice. An in vitro packaging reaction was

20   carried out using a Giga Pack II Gold (Strotagene), and 5.34×10^6 pfu of recombinant phages were obtained from the above-mentioned 3 ligase reaction products. The library was amplified in E. coli Y1090 r^{-1} host to obtain a stock of 1.7×10^{11} pfu/ml HPC-Y11 phage library.

25   C.   Identification and isolation of 5'-portion of megakaryocyte differentiation factor cDNA

(1)   Amplification by PCR of HPC-Y11 phage library cDNA insert DNA fragment primer NI074 upstream portion.

30   To 1 µl (corresponding to

1.7×10^9 pfu) of 6.0×10^{10} pfu/ml HPC-Y11 phage library
stock solution as a template DNA for PCR reaction, were
added 5 µl of 10 × PCR buffer, 8 µl of 1.25 mM 4 dNTPs,
1 µl of 10 OD/mlλgt11-forward F1 primer (SEQ ID NO: 25),
1 µl of 50 D/ml NI074 primer and 1 µl of Perfect Match
(Stratagene), and a total volume was made to be 49 µl
with DEPC-treated distilled water.

After heating the reaction mixture at
95°C for 5 minutes and 60°C for 5 minutes, 1 µl of 5 U/µl
Taq DNA polymerase (Perkin Elmer Cetus) was added
thereto, and 35 reaction cycles of 94°C for a minute,
60°C for a minute and 72°C for 2 minutes were carried
out, followed by incubation at 72°C for 10 minutes. A
result of analysis by 2% agarose gel electrophoresis
showed a smeary pattern ranging from 0.3 to 6 kb.

(2) PCR analysis using a template which
is a PCR amplification fragment mixture prepared by using
λgt11F1/NI074 primer, and using as primers λgt11F2 (SEQ
ID NO: 26)/NI075, λgt11F2/TP12 (SEQ ID NO: 28;
corresponding to 703-683 of SEQ ID NO: 30), λgt11F2/TP11
(SEQ ID NO: 27; corresponding to 619-599 of SEQ ID
NO: 30), λgt11F2/TP13 (SEQ ID NO: 29; corresponding to
595-575 of SEQ ID NO: 30), TP7/NI074, TP7/NI075, and
NI073/NI074

1 µl of a 1/100 diluted solution of
the above-mentioned PCR reaction product was used for a
template DNA for PCR reaction, and 5 µl of 10 × PCR
buffer, 8 µl of 1.25 mM 4 dNTPs, 0.5 µl each of 10 OD/ml
primers in combination as described hereinafter, and 1 µl
of Perfect Match (Stratagene) were added thereto, and a

total of the reaction mixture was made to be 49 µl with
DEPC-treated distilled water. The reaction mixture was
heated at 95°C for 5 minutes and at 60°C for 5 minutes,
1 µl of 5 U/µl Taq DNA polymerase (Perkin Elmer Cetus)
was added thereto, and 35 reaction cycles of 94°C for a
minute, 60°C for 2 minutes and 72°C for 2 minutes were
carried out, followed by an incubation at 72°C for
10 minutes.

As primers, λgt11F2/NI075,
λgt11F2/TP12, λgt11F2/TP11, λgt11F2/TP13, TP7/NI074,
TP7/NI075, and NI073/NI074 were used. As a result of 2%
agarose gel electrophoresis, bands of 969 bp, 870 bp,
786 bp, 762 bp, 330 bp, 120 bp and 149 bp were obtained
corresponding to the primers.

(3) PCR analysis and primary sequence
analysis using λgt11F/TP11 and λgt11F/TP13 primer, for
PCR amplification product (969 bp) prepared using
F2/NI075 primers

0.5 µl of the above mentioned PCR
reaction product (969 bp) prepared by using λgt11F2/NI075
as primers was used as a template DNA for PCR reaction,
and 5 µl of 10 × PCR buffer, 8 µl of 1.25 mM 4 dNTPs,
1 µl of 10 OD/ml λgt11F primer, 1 µl of 10 OD/ml TP11
primers or 1 µl of 10 OD/ml TP13 primers, and 1 µl of
Perfect Match (Stratagene) were added, and the total
volume was made to be 49 µl with DEPC-treated distilled
water.

The reaction mixture was heated at
95°C for 5 minutes and 60°C for 5 minutes, 1 µl of 5 U/µl
Taq DNA polymerase (Perkin Elmer Setus) was added

thereto, and 35 reaction cycles of 94°C for a minute, 60°C for 2 minutes and 72°C for 2 minutes were carried out, followed by an incubation at 72°C for 10 minutes. Bands of PCR reaction products

5　(678 bp and 654 bp, respectively) were excised from 2% agarose gel after electrophoresis, extracted and purified, and inserted into pCRII (Invitrogen), which was then used to transform E. coli IN VαF' (Introgen). Plasmid DNA was extracted and purified from the

10　transformant, and digested with EcoRI to confirm that a 0.7 kb DNA fragment was inserted. The primary sequence of the inserted DNA fragment was analyzed by using M13 forward primer M13F and M13 reverse primer M13R (Applied Biosystems automated sequencer Model 370A).

15　As a result, a sequence of 619 bp corresponding to nucleotide number 1 to 619 of SEQ ID NO: 30 was found, and the nucleotide sequence of 133 nucleotides consisting of nucleotide number 487 to 619 of SEQ ID NO: 30 conformed to the N-terminus of the primary

20　sequence found in Example 2.1. In this 619 bp sequence there are 19 amino acids of SEQ ID NO: 3 (VERVDFTNHLEDTR RNINK from DNA nucleotide sequence) and 5 amino acids (LYDAK) of SEQ ID NO: 7, and it was clarified that this PCR reaction products (each 0.7 kb) was a part of cDNA

25　coding for megakaryocyte differentiation factor. It was considered that the translation starting methionine corresponds to 74th nucleotide, and 5'-non translational region consisted of 73 bp. Accordingly, it was clarified that these PCR

30　reaction products (each 0.7 kb) contain N-terminus of structural gene for megakaryocyte differentiation factor.

5.   Analysis of cDNA nucleotide sequence coding for
megakaryocyte differentiations factor by PCR (4)

Among an N-terminal portion of structural gene
for a megakaryocyte differentiation factor derived from
HPC-Y11 and a sequence considered to be a 5'-non-
translational region, obtained in the section C(3), the
sequence of nucleotide numbers 12 to 31 of SEQ ID NO: 30
which is a sequence part considered to be a 5'-non-
translational region was used as a basis to synthesize an
oligomer NI 083 (SEQ ID NO: 31).

The RNA having poly A prepared from A431 cells
in the section 1., a Preamplification System (Gibco BRL)
and random hexamers attached to the System were used to
synthesize first strand cDNA according to included
instructions, and PCR was carried out by using NI083 and
NI074 as well as Ampli Taq (Takara). As a result, a DNA
fragment of 1001 bp which is a cDNA fragment for a
megakaryocyte differentiation factor was obtained.

This DNA fragment, a PCR product, of 1001 bp
was directly used as a substrate for sequencing on a Taq
Dye Deoxy Terminator Cycle Sequencing kit (Applied
Biosystem) and a fluorescent sequencer (Applied Biosystem
Type 370A) according to the included manufacturers
instruction. As a result, a sequence of nucleotide
numbers 32-486 of SEQ ID NO: 30 was found. In addition,
a result obtained for nucleotide numbers 487 to 991 of
SEQ ID NO: 30 conformed to the sequence obtained in the
section 1.

By combining with the sequence obtained in the
section 1., the nucleotide sequence 32-1950 of SEQ ID
NO: 30 which is cDNA nucleotide sequence coding for a

megakaryocyte differentiation factor of A431 cell was determined.

For this nucleotide sequence, all of possible 3 reading frames were mechanically translated into amino acid sequences and it was found that one of them has a region which can be translated to a continuing amino acid sequence containing all the amino acid sequences shown in SEQ ID NO: 1 to 9, and the reading frame of the megakaryocyte differentiation factor was determined.

It was found that in this reading frame a codon for methionine (nucleotide numbers 74 to 76) found at a translation start position exists, and from this position a region which can be translated to an amino acid sequence containing the amino acid sequences shown in SEQ ID NO: 1 to 9 continues up to the nucleotide position 1213 of SEQ ID NO: 30, and it was determined that the nucleotide sequence from the position 74 to the position 1213 of SEQ ID NO: 30 is the region translated to megakaryocyte differentiation factor.

The nucleotide sequence GCAATGG (nucleotide numbers 71 to 77 of SEQ ID NO: 30) encompassing the methionine codon of nucleotide numbers 74 to 76 of SEQ ID NO: 30 corresponds to a sequence (G/A-N-N-A-T-G-G) encompassing a methionine codon frequently present at a translation start site found by M. Kozak, Nucleic Acids Research (1981) Vol. 9, p5233-5252.

Primary sequence of megakaryocyte differentiation factor was thus clarified, the number of amino acids was 380 in the structural gene, an expected molecular weight was 42904.43, and an expected isoelectric point was 6.79. SEQ ID NO: 1 corresponds to

amino acid numbers 188 to 196 of SEQ ID NO: 30; SEQ ID
NO: 2 corresponds to amino acid numbers 181 to 187 of SEQ
ID NO: 30; SEQ ID NO: 3 corresponds to the amino acid
numbers 126 to 144 of SEQ ID NO: 30; SEQ ID NO: 4

5   corresponds to the amino acid numbers 325 to 341 of SEQ
ID NO: 30; SEQ ID NO: 5 corresponds to the amino acid
numbers 289 to 297 of SEQ ID NO: 30; SEQ ID NO: 6
corresponds to the amino acid numbers 305 to 324 of SEQ
ID NO: 30; SEQ ID NO: 7 corresponds to the amino acid

10   numbers 121 to 125 of SEQ ID NO: 30; and SEQ ID NO: 8
corresponds to the amino acid numbers 284 to 288 of SEQ
ID NO: 30; and SEQ ID NO: 9 corresponds to the amino acid
numbers 204 to 213 of SEQ ID NO: 30. In addition, A poly
A addition signal AATAAA sequence is present at

15   nucleotide numbers 1933 to 1998 of SEQ ID NO: 30.

Example 3. Isolation and identification of cDNA
coding for megakaryocyte differentiation factor from A431
by PCR and construction of expression vector

Oligomers NI078 (SEQ ID NO: 32) and NI079 (SEQ ID

20   NO: 33) were synthesized on the basis of the sequence
(SEQ ID NO: 30) obtained in Example 2. Note in the
NI078, the sequence of nucleotide numbers 13 to 37
encompassing the translation start methionine codon
conforms to the sequence of the nucleotide numbers 74 to

25   98 of SEQ ID NO: 30 and an EcoRI recognizing site
(nucleotide numbers 4 to 9) and an Nru I recognizing site
(nucleotide numbers 8 to 13) were artificially added; and
in the NI079, the sequence of the nucleotide numbers 17
to 49 conforms to the nucleotide sequence of the

30   nucleotide numbers 1237 to 1269 of SEQ ID NO: 30, and an
EcoRI recognizing site (nucleotide numbers 3 to 8) and an

Not I recognizing site (nucleotide numbers 9 to 16) were artificially added.

The RNA having poly A prepared from A431 cells in Example 2 section 1., a Preamplification System (Gibco

5   BRL), and Oligomers, i.e., random hexamers included in the system were used according to the included manufacturer's instructions to synthesize first strand cDNA, and PCR was carried out using the synthesized DNA as a template and NI078 and NI079 as primers and using

10   Ampli Taq (Perkin Elmer Cetus). As a result, a DNA fragment of 1224 base pairs which is a cDNA fragment for a megakaryocyte differentiation factor and has all information relating to megakaryocyte differentiation factor, was obtained.

15   This DNA fragment was treated with EcoRI to generate EcoRI cohesive sites at both the ends of the cDNA coding for a megakaryocyte differentiation factor in virtue of EcoRI recognizing sites artificially added to the oligomers NI078 (SEQ ID NO: 32) and NI079 (SEQ ID

20   NO: 33). This cDNA fragment coding for megakaryocyte differentiation factor was introduced into a mammorian expression vector pdKCR-DHFD at it's EcoRI recognizing site to obtain pdKCR-DHFR-TPO55.

The animal cell expression vector pdKCR-dhfr

25   (Oikawa, S. et. al., Biochem. Biophys. Res. Commun. 164, 39, 1989) is a derivative of pKCR (O' Hare et. al., Pro. Natl. Acod. Sci. USA, 78, 1527, 1981) and has SU 40 early promoter and a rabbit ß-globin gene and dhfr (dehydrofolate reductase) gene. Note, a host transformed

30   with the expression vector, was designated as <u>Escherichia</u> <u>coli</u> SBM 308, and deposited with the Fermentation

Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 3-chome, Tsukuba-shi, Ibaraki, Japan as FERM P-11506 on June 7, 1990, and transferred to an international deposition under the Budapest treaty as FERM BP-4197 on February 18, 1993.

The clone pdKCR-DHFR-TPO55 containing megakaryocyte differentiation factor cDNA which was incorporated in to pdKCR-DHFR was sequenced using a Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystem) and a fluorescent sequencer (Applied Biosystem Type 370A) according to included instructions. As a result, the determined nucleotide sequence conformed to the sequence of the nucleotide numbers 99 to 1236 of SEQ ID NO: 30 and oligomers NI078 and NI079. In addition, it was confirmed by the sequencing that a megakaryocyte differentiation factor cDNA inserted into the vector is in correct orientation in relation to an expression vector promoter.

As shown in the above, once the information of SEQ ID NO: 30 is provided, it is easy for a person skilled in the art that the nucleotide sequence is determined by amplifying cDNA coding for megakaryocyte differentiation factor in total or in a optional portion on megakaryocyte differentiation factor expressing cell line (for example, A431) and boned in a optional expression vector.

Example 4. Expression of megakaryocyte differentiation factor in Bombyx mori

(1) Construction of Bombyx mori expression vector

A megakaryocyte differentiation factor cDNA clone pdKCR-DHFR-TP055 was digested with NotI to cleave the NotI recognizing site artificially added to the NI079. The NotI cohesive end thus generated was blunt-

ended using a blunting kit available from Takara Shuzo, and to the blunt end was added an XbaI linker (Takara Shuzo) according to an attached instruction. The plasmid thus obtained was digested simultaneously with NruI and

5 XbaI to cleave the NruI recognizing site artificially added to the NI078 and the XbaI recognizing site of the XbaI linker introduced to prepare a megakaryocyte differentiation factor cDNA fragment having an NurI cohesive end and an XbaI cohesive end. This DNA fragment

10 was inserted at the Nru I recognizing site into a baculovirus transfer vector for <u>Bombyx</u> <u>mori</u> nuclear polyhedrosis virus, pBm4 (available from Department of Insect Genetics and Bleeding National Institute of Sericultural and Entomological Science, Ohwashi, Tukuba,

15 Ibaraki 305, Japan) simultaneously digested with NruI and XbaI to obtain pBm4-TPO55.

(2)   <u>Construction of TPO55 recombinant virus</u>

A cell line derived from <u>Bombyx</u> <u>mori</u> embryonic, BoMo15AIIc (available from Department of Insect Genetics

20 and Bleeding National Institute of Sericultural and Entomological Science, Ohwashi, Tukuba, Ibaraki 305, Japan) was subcultured in a medium containing 10% fetal bovine serum (FBS: GIBCO BRL) and 500 μg/ml gentamicin in MGM 448 at 25°C. TPO55 recombinant virus was

25 constructed by co-introducing <u>Bombyx</u> <u>mori</u> nuclear polyhedrosis virus gene DNA and pBm4-TPO55 plasmid DNA into <u>Bombyx</u> <u>mori</u> cultured cells by, for example, calcium phosphate co-precipitation method.

Namely, 2 μg of genomic DNA of wild type virus

30 B6E (available from Department of Insect Genetics and Bleeding National Institute of Sericultural and

Entomological Science, Ohwashi, Tukuba, Ibaraki 305,
Japan) and 10 µg of the transfer plasmid pBm4-TPO55 were
dissolved in 240 µl of sterile purified water, and to the
solution was added the same volume of 0.5 M $CaCl_2$ and

5   0.1M HEPES, and the mixture was mixed and allowed to
stand at a room temperature for 10 minutes. To the
mixture was added 480 µl of 0.2M NaCl, 0.05M HEPES,
0.75 mM NaH_2PO_4 and 0.75 mM Na_2HPO_4, and the mixture was
stirred for a few second and allowed to stand at a room

10   temperature for 20 to 30 minutes to form calcium
phosphate gel containing the genomic DNA and the plasmid.
        Next, 960 µl of calcium phosphate gel
suspension containing the viral genomic DNA and the
transfer vector was added to 4 ml of BoMo15AIIc cells in

15   a 25 cm² T flask (T25, Corning), and the mixture was
allowed to stand for 12 hours. The medium was replaced
with a fresh MGM448 (containing 10% FBS and the
antibiotics), and culturing was carried out for 25°C. On
the sixth day the cultured medium was recovered as a

20   viral solution.
        The cultured medium was centrifuged to obtain a
clear supernatant, which was then diluted, and added to
BoMo15AIIc cells cultured on the microtiter plate, and
after 8 days a culturing medium in which viral infection

25   was microscopically observed but a polyhedral body was
not formed, was selected (by a limited dilution method).
The cultured medium was recovered. Contamination with a
wild virus in the viral solution factor was not observed.
        A recombinant virus thus constructed,

30   containing a DNA coding for megakaryocyte differentiation
factor was designated as TPO55-BmNPV.

(3) Test for expression of recombinant gene

About 1×10^6 BoMo15A IIc cells were cultured in 4 ml of MGM448 medium containing 10% FBS on the bottom of 25 cm² area of a flask for 2 days by plate culture. To the culture, 0.5 moi of wild type virus B6E or recombinant virus (TPO55-BmNPV) containing a gene coding for megakaryocyte differentiation factor were added and infected, BoMo15A IIc cells, and the cells were cultured at 25°C for 3 days, and total RNA was extracted using Isogen (Wako Pure Chemical). Similarly, total RNA was extracted from non infected BoMo 15AIIc cells.

Next, 1 Mg of the RNA thus extracted was size-fractionated by agarose gel electrophoresis, and the separated RNA was transferred to a Zetaprobe menbrane by the capillary action. The menbrane was soaked in a hybridization buffer containing megakaryocyte differentiation factor cDNA (:PCR product amplified with KY100 and NIO65 described in Example 2.1) (TPO55 probe DNA) labeled with digoxigenin (Boehringer Mannheim), and the mixture was incubated at 42°C for 12 hours to allow the formation of specific complex of recombinant megakaryocyte differentiation factor mRNA and the TPO55 probe DNA thereof. The complex was then reacted with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim), and the complexed megakaryocyte differentiation factor mRNA was detected by chemoluminescence generated by hydrolysis of Lumigen PPD (AMPPD) (Boehringer Mannheim) according to manufacture's instructions with alkaline phosphates.

As seen in Fig. 11, recombinant megakaryocyte differentiation factor mRNA was detected in total RNA

extracted from TPO55-BmNPV-infected cells, and it was shown that the mRNA was expressed in the TPO55-BmNPV-infected cells. On the other hand expression of mRNA which hydridized with the probe DNA was not observed in the B6E-infected cells and un-infected cells.

(4) Preparation of solution of recombinant virus

About 1×10^6 BoMo15AIIc cells were cultured in 4 ml of MGM448 containing 10% FBS on the bottom of a 25 cm² flask for 2 days, and to this culture was added 10 µl of the culture medium of BoMo15AIIc cells containing the recombinant virus cloned in the above section (2). After culturing at 25°C for 14 days, the culture medium was centrifuged at 1000 rpm for 5 minutes to obtain a supernatant as a recombinant virus solution.

(5) Preparation of hemolymph of Bombyx mori

50 µl/head of a viral solution of the 10^{-1}-diluted recombinant virus solution obtained in the above section (3) or a 10^{-1}-diluted wild type virus B6E solution was injected to Bombyx mori larvae at 5th instar, and the silkworms were fed with commercially available artificial feed (Morus; Katakura Kogyo) at 20°C for 4 to 5 days. The abdomens of 50 silkworms were cut, and an extract containing the hemolymph and the content in the central intestine was taken in a plastic tube cooled with ice, and centrifuged to obtain a supernatant.

(6) Confirmation of activity of megakaryocyte differentiation factor

50 ml of the hemolymph of the silkworms obtained in the above section (5) was thoroughly dialyzed against a 20 mM Tris/HCl (pH 7.4) buffer, and applied to a Matrex Blue A column (ϕ2.5 × 15 cm) equilibrated with

the same buffer. The column was thoroughly washed with the same buffer to eliminate a unbound fraction, and a bound protein was eluted by a concentration gradient of 0 to 1M NaCl. An elution profile for megakaryocyte

5   differentiation activity of the hemolymph obtained from silkworms injected with the recombinant virus was compared with that for a wild type virus.

As seen from Fig. 12, megakaryocyte differentiation activity in the hemolymph from silkworms

10   injected with the recombinant virus was significantly higher than that for the wild type virus.

Although Bombyx mori vacurovirus transfer vector pBm4, Bombyx mori nuclear polyhedrosis virus PbE and Bombyx mori cells BoMo15AIIc were used in Example 4,

15   the present invention is not limited to the use of these materials. Namely, other baculovirus transfer vector (such as pBK283, pBKblue, available from Funakoshi) Bombyx mori nuclear polyhedrosis virus (such as purified DNA available from Funakoshi), Bombyx mori cells (such as

20   BmN4 cells, available from Funakoshi) can be easily used by a person with ordinary skill in the art to obtain a megakaryocyte differentiation factor.

A megakaryocyte differentiation factor of the present invention accelerates formation of megakaryocytes

25   from myeloid cells in the presence of IL-3. The present megakaryocyte differentiation factor plays an important role in differentiation of megakaryocytes and acts in vivo as a thrombopoietin. Accordingly, the present megakaryocyte differentiation factor may be medicaments

30   effective to not only various diseases involving decrease of platelets but also for control of the number of

platelets decreasing by radiation in the case of bone marrow transradiation, or for control of the number of platelets in chemotherapy of cancers.